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CONTRACTING ORGANIZATION: Mayo Foundation
Rochester, Minnesota 55905

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13. ABSTRACT ***(Maximum 200 Words)***

The metabolites of estrogens, the catecholestrogens (CEs), can be activated to form stable and depurinating DNA adducts through a series of reactions. The adducts formed from these CEs, namely 4- hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2), result in mutations that lead to genotoxicity and therefore breast carcinogenesis. Prevention of the genotoxic effects can be achieved in part through the sulfate-conjugation of the CEs, catalyzed by sulfotransferase (SULT) enzymes. Because many of the human SULTs are genetically polymorphic, inherited differences in activities of these enzymes may contribute to the pathophysiology of breast cancer. We determined the activity of 13 recombinant human SULTs with 4-OHE1, 4-OHE2, 2- hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), estrone (E1) and 17-β estradiol (E2). SULT1E1 had the highest affinity for them all, with apparent Km values of 0.31 and 0.18 uM for 4-OHE1 and 4-OHE2. We also performed immunohistochemical studies with SULT1E1 antibody and determined the presence of SULT1E1 in breast tissue block arrays of non-cancer and tumor samples. We then resequenced the SULT1E1 gene with DNA from 60 Caucasians and 60 African-American subjects and identified 3 nonsynonymous cSNPs that changed encoded amino acids: Asp22Tyr, Ala32Val and Pro253His. Functional genomics studies showed that 2 of the 3 nonsynonymous cSNPs had decreased enzyme activities with corresponding decreases in immunoreactive protein. These observations suggest that ethnic-specific variations in sulfation of CEs catalyzed by SULT1E1 may contribute variable risk to the development and pathophysiology of estrogen-dependent diseases such as breast cancer.

14. SUBJECT TERMS
Breast Cancer, Sulfation, Sulfotransferases, nonsynonymous cSNPs, SULTs genetic polymorphisms, Catecholestrogens, Immunohistochemical studies, Resequence

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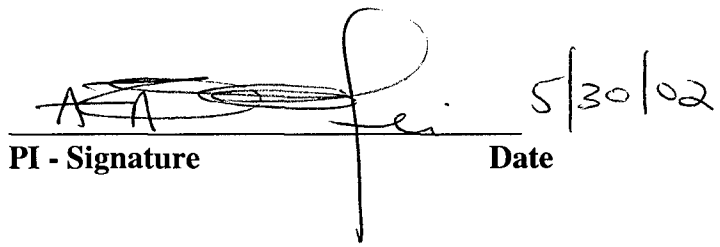

5/30/02

PI - Signature **Date**

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INTRODUCTION

A growing body of evidence suggests that the carcinogenic effect of estrogens may also result from nonreceptor -mediated mechanisms with estrogen-metabolites as direct genotoxins. Prevention of the genotoxic effects of the estrogens, as a result of formation of depurinating adducts by the catecholestrogens (CEs), 4- hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2), can be achieved in part through inactivation of the CEs, in reactions catalyzed by sulfotransferase (SULT) enzymes. Many of the human cytosolic SULTs are genetically polymorphic. Thus, inherited differences in the activities of these enzymes would lead to variations in enzyme activities and variations in the inactivation of the CEs. We determined that most of the SULTs can inactivate the CEs through sulfate -conjugation. We also observed that SULT1E1 had the highest affinity for the CEs. We then localized SULT1E1 in breast tissue from non-cancer and tumor samples. We also determined that the SULT1E1 gene contained three single nucleotide polymorphisms (SNPs), two of which had low enzyme activities and levels of immunoreactive protein. These variations imply that individuals with those SNPs may show increased formation of the genotoxins which, may ultimately contribute to the individual's risk of developing estrogen-dependent diseases such as breast cancer.

BODY

Task 1 was to determine which of the known human SULT isoforms and allozymes are capable of catalyzing the sulfate –conjugation of the catecholestrogens 2-OHE1, 2-OHE2, 4-OHE1 and 4-OHE2. Recombinant SULT proteins developed in our laboratory was used for the Substrate Kinetic studies. A latter addition to this Task was the inclusion of the parent estrogens, Estrone (E1) and Estradiol (E2), whose metabolites are the CEs. 1-6 months.

This Task is completed. A modification of the method of Foldes and Meek for assaying SULTs was used for these substrate kinetic studies.
Two Abstracts and a Manuscript are attached which describe this work.

Task 2 was to use immunohistochemical techniques to localize the SULT isoform(s), which show relatively high affinities for the CEs, in normal and neoplastic breast tissues. 6-12 months.

This task is completed.
SULT1E1 antibody was used to perform immunohistochemical assays. Breast tissue block arrays, produced by Dr. Patrick Roche, a co-investigator on this award, in the Experimental Pathology and Immunostaining laboratory, were used for the immunohistochemistry studies. The tissue block arrays consisted of breast tumor tissues and non-cancer breast tissues. Approximately 93% of the breast tumor tissue samples expressed SULT1E1 compared to about 57% of the non-cancer tissues. When stratified for estrogen receptor status (ER+ and ER-), results from our cohort of samples indicated that approximately 85% of the tumors were ER+, while only 15% were ER-. All of the

ER- tumors expressed SULT1E1 protein, while only 92% of the ER+ tumor samples stained positively with the SULT1E1 antibody. Of the non-cancer tissue samples that expressed SULT1E1 approximately 71% of them were ER+.
[Detailed results in Annual report 2001]

Task 3 was to test the hypothesis that there are functionally significant genetic polymorphisms within the specific SULT gene(s) identified and to develop allele-specific restriction digestion assays for these sequences to enable rapid genotyping. 7-24 months.

This task is completed.
DNA samples from the Coriell Institute was used for this part of the studies. This DNA, comprised a subset of a 100 sample from Caucasian- American and of a 100 sample African- American DNA set. These DNA samples had been striped of all other identifiers. It is of great importance in today's research to acknowledge the ethnic background of DNA used in pharmacogenetics studies. For example, valuable information with regards to allele types and frequencies in the different ethnic groups that may have important biological and or medical consequences could be missed if ethnic variations are ignored. Therefore, in this case, to obtain meaningful data with regards to types of alleles and their frequencies, we resequenced120 DNA samples, 60 from Caucasian- American and 60 from African- American subjects. All eight (8) exons of the SULT1E1 gene were amplified from each of the 120 DNA samples. Twenty-three (23) single nucleotide polymorphisms (SNPs) were identified, including 3 nonsynonymous cSNPs which changed the following encoded amino acids: Asp22Tyr and Ala32Val in exon 2 and Pro253His in exon 7. The Asp22Tyr was found in an African-American DNA sample, Ala32Val and Pro253His were detected in Caucasian DNA samples.
Functional genomic studies performed with the allozymes showed that the Asp22Tyr variant and the Ala32Val variant had reduced basal enzyme activity levels when compared to that of the wild type enzyme. Based on the three nonsynonymous cSNPs detected,
allele-specific restriction digestion assays were developed to enable rapid genotyping of other DNA samples.
[Details for Task 3 in 2001 annual report].

Excerpt from the 2001 Annual Report, under Key Research accomplishment:

We performed one type of genotype-phenotype correlation, i.e. comparing the functionally important genotypes with levels of enzyme activity. We are repeating a second phenotype correlate i.e. determining quantity of immunoreactive protein, as an additonal important information for Task 3. Finally, we are validating the

allele-specific digestion assay using the specified restriction enzymes

To complete Task 3, the functional genomic studies were repeated for the three allelic variants observed during the resequencing.
Transfections using a new batch of COS-1 cells were performed with expression constructs created for each of the variant SULT1E1 alleles. The Transfast method of transfection was used. The recombinant proteins obtained were measured for basal SULT1E1 activity corrected for transfection efficiency in reactions that used 17-β-Estradiol as the substrate. The resulting activities, described as percentages of wild type SULT1E1 activity are shown in Figure 1. The Asp 22Tyr showed about 13% the activity of the wild type, Ala32Val showed 56% of the wild type activity and the Pro253His showed an activity level that was similar to the wild type activity.
We then performed Western Blot analysis with the recombinant proteins from the allozymes to determine the levels of immunoreactive protein. Both Asp 22Tyr and Ala 32Val showed reduced levels of immunoreactive protein that correlated with the reduced enzyme activities (Figures1 and 2).
Substrate kinetic studies were also performed for the recombinant allozymes, using 17-β-estradiol as the prototypic substrate and 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the co-substrate. The apparent Km values are shown in Table 1. The reduced activity observed for the Asp22Val could be explained as due to both reduced levels of immunoreactive protein and higher Km values (greater than 5 fold) for both substrates as compared to the wild type enzyme (Table 1, Figures 1 and 2). The reduced activity seen for the Ala32Val allozyme was due in part to the reduced level of immunoreactive protein (Table 1, Figures 1 and 2). Although Pro253His showed basically the same enzyme activity and immunoreactive protein as the wild type, there was a 3-fold increase in Km values for 17-β-estradiol and PAPS (Table 1, Figures 1 and 2).

These results are in a manuscript being prepared for publication.

An ABSTRACT pertaining to this work can also be found in the appendix.
This Abstract was chosen for an oral presentation and was also presented as a Poster at the American Society for Clinical Pharmacology and Therapeutics (ASCPT) annual meeting held in Atlanta, Georgia, in March 2002.
This Abstract received the 2002 Presidential Award for the Society.

Task 4 : To compare the allele frequencies for the genetically polymorphic SULT isoform(s) identified in specific aim 2, in an age-matched control population and patients with breast cancer.
This Task has been completed

Based on the resequencing data detailed in the 2001Annual Report (also see Table 2), allele-specific digestion assays were developed to genotype DNA from women participating in the Minnesota Breast Cancer family Study under the ongoing Breaast Cancer Program Project Grant (PPG) here at Mayo. It is of great importance to state here

that all IRB requirements for this PPG have been met. The total number of DNA samples assayed so far is 1120.

Primers were designed to amplify exons 2 and 7 for assays based on restriction sites that were introduced into the DNA sequence by the variant nucleotides. For the Asp22Tyr change, an additional *Ssp1* site was introduced in the DNA segment as a result of the SNP therefore, an *Hpy188 III* restriction site was incorporated in the primer design for digestion of the wild type sequence. For the Ala32Val change, an *Mwo1* site was removed by introduction of the SNP. The wild type sequence variant could then be digested with *Mwo1*. Because the Pro253His change did not introduce any new restriction site in exon 7, a Dra III site was incorporated in the primers designed to ampilfy exon 7. Dra III would digest the variant DNA.

PCR amplifications were performed for each of the samples using primers designed to amplify the exons. Digestion reactions for each of the amplicons were performed with 10 Units of enzyme. All of the digested reactions were run on 3% agarose gel. Control digestions were performed with DNA samples that had been genotyped for the heterozygous alleles(which has both the variant and wild type genotypes) and wild type samples determined initially from the resequencing study. The results indicated the absence of any of the SNPs in these samples. It is important to note that the resequencing results indicated an allele frequency of only 0.8% for each of the SNPs. Therefore, for meaningful results, a much larger sample size than what was used in this study would be required to detect these SNPs.

KEY RESEARCH ACCOMPLISHMENTS

- We determined that catecholestrogens are substrates for most of the SULTs and that SULT1E1 had the highest affinity for 4-OHE2, the CE responsible for forming the genotoxins that form carcinogens.

- In our immunohistochemical studies using Breast Tissue Block arrays we have shown that SULT1E1 is expressed in breast tissues of both non- cancer and tumor samples.

- We resequenced SULT1E1 gene in 120 DNA samples and analyzed approximately 730 kb of sequence. We identified 23 SNPs, 18 in African-American subjects and 13 in Caucasian -American subjects from this resequencing study. Only 3 SNPs in the coding region of SULT1E1 gene changed encoded amino acids.

- We have used a genotype-to- phenotype strategy to identify variant alleles of SULT1E1 that are of functional importance. The genotypes correlate with levels of enzyme activity and with levels of immunoreactive protein.

- We have used allele-specific digestion assays to genotype a cohort of women in the Minnesota Breast Cancer Family Study. Results from this study suggest that a larger sample size is needed to access any possible contribution of these SULT1E1 SNPs to risk for breast cancer.

REPORTABLE OUTCOMES

Abstracts/Posters/Talks/Manuscript

1. Adjei, A.A., Wood, T.C., Roche, P.C. and Weinshilboum, R.M.: Sulfate conjugation and estrogen-mediated breast carcinogenesis. Clin. Pharmacol. Ther. 67:2, 140, 2000.
- Abstract won the 2000 Presidential Trainee Award, American Society of Clinical Pharmacology and Therapeutics(ASCPT) Presidential Award
- Also presented as a Talk at the ASCPT Meeting

2. Sulfate Conjugation and Estrogen-Mediated Carcinogenesis.
Araba A. Adjei, Thomas C. Wood, and Richard M. Weinshilboum.
Department of Molecular Pharmacology and Experimental Therapeutics.
Mayo Clinic, Rochester, MN.
- Poster presented at the Mayo Women's Breast Cancer Program Symposium, 2000

3. Adjei, A.A., Thomae, B.A., Prondzinski, J.L., Eckloff, B., Weiben, E. and Weinshilboum, R.M.: Human Estrogen Sulfotransferase (SULT1E1) Pharmacogenetics: Gene Resequencing and Functional Genomics. Clin. Pharmacol. Ther. 71:2, 40, 2002.
- Abstract won the 2002 Presidential Trainee Award, American Society of Clinical Pharmacology and Therapeutics(ASCPT) Presidential Award
- presented as a Poster at the ASCPT Meeting
- presented as a Talk at the ASCPT Meeting

4. Adjei Araba A. and Weinshilboum Richard M. Catecholestrogen Sulfation: Possible Role in Carcinogenesis
Biochemical and Biophysical Research Communications 292: 402-408, 2002.

5. A manuscript entitled Human Estrogen Sulfotransferase (SULT1E1) Pharmacogenetics: Gene Resequencing and Functional Genomics is in preparation.

6. A Patent application for the SULT1E1 SNPs detected during the resequencing is being submitted.

CONCLUSIONS

As a step towards determining whether sulfate-conjugation catalyzed by SULTs may represent an independent risk factor for the development of breast cancer, we have determined that the catecholestrogens are substrates for most of the SULTs.
We also determined that SULT 1E1 had the highest affinity for 4-OHE2, the CE responsible for forming the genotoxins that form carcinogens. We have also shown that SULT1E1 is present in breast tissue of both cancer and non-cancer breast tissue.
Moreover we have been able to show that, like other SULTs, SULT1E1 is genetically polymorphic and has 2 polymorphisms; Asp22Tyr and Ala32Val that are of functional significance.

SO WHAT?

Sulfate -conjugation catalyzed by SULTs is one of the major important pathways in the biotransformation of drugs, xenobiotics, neurotransmitters and steroid hormones. From our report, the majority of the SULTs known to date will inactivate the catecholestrogens (CEs) to various extents. This inactivation, if our hypothesis holds true, would reduce the genotoxic effect that might occur when these reactive estrogen metabolites react with DNA. As such, these results from our proposal/ project show that there is variation in SULT1E1 activity that is due to genetic polymorphisms. These polymorphisms are of functional significance such that variations in enzyme activity (as seen using estradiol as the prototypic substrate) would also indicate variations in the inactivation of the CEs that form reactive quinones and interact with DNA to form genotoxins. These low activity variant allozymes would inactivate the CEs to a lesser extent. Therefore patients with these low activity variants might be at an increased risk of developing genotoxic conditions that might lead to cancer.

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APPENDICES

Appendix I :

FIGURES AND TABLES.

Figures

Figure 1. Functional Genomics: Recombinant SULT1E1 Allozyme Activity.

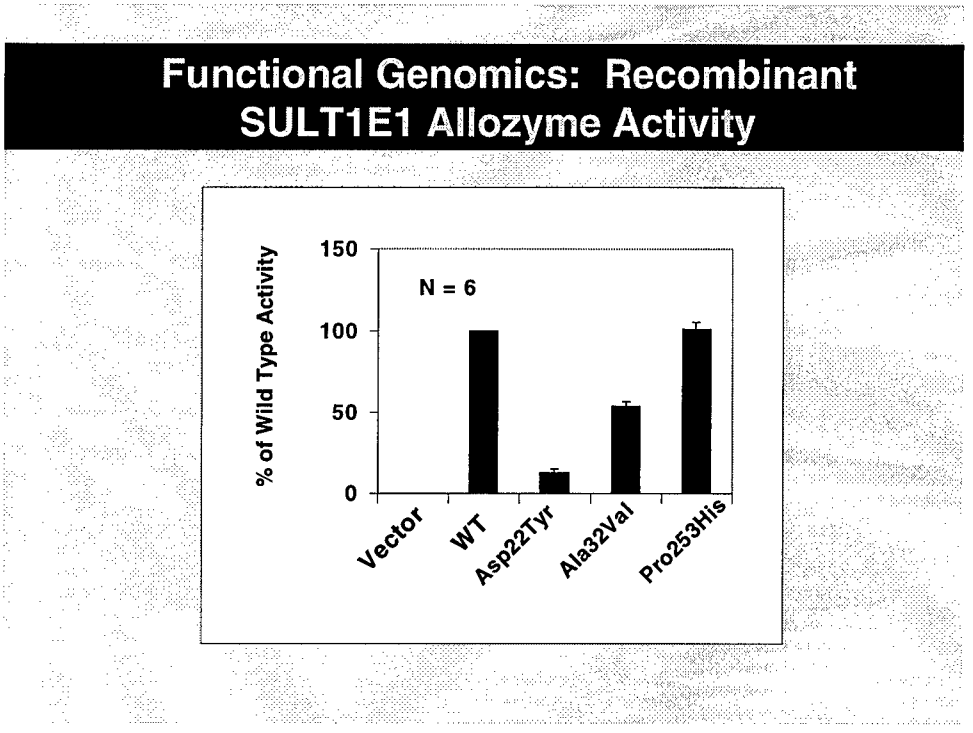

Figure 2. Functional Genomics: SULT1E1 Immunoreactive Protein

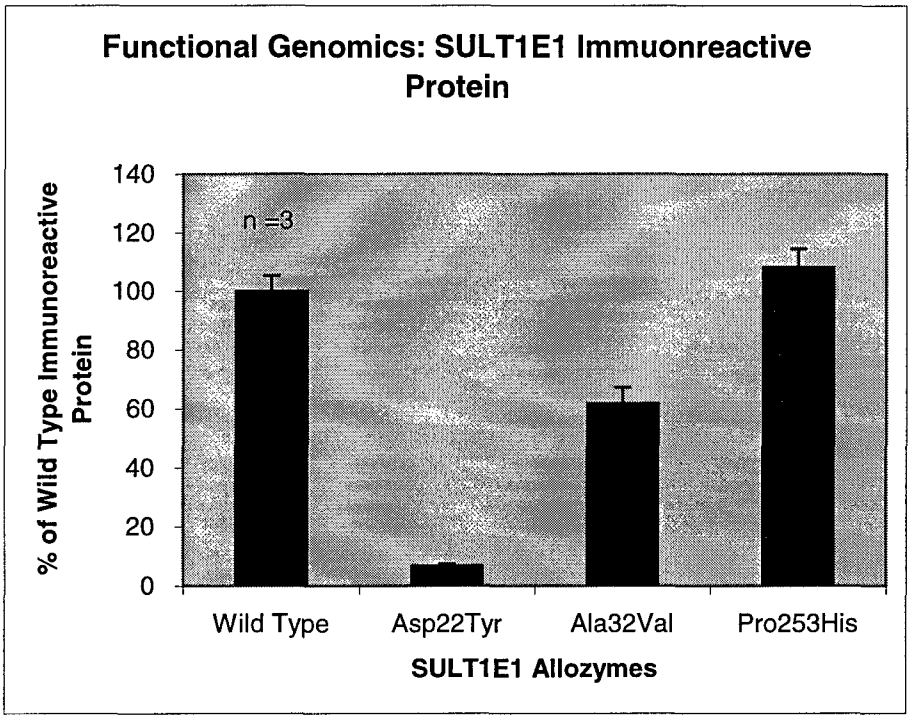

Table 1. Substrate Kinetics: Apparent Km Values for Recombinant SULT1E1 Allozymes with Estradiol and PAPs as Substrates.

Substrate Kinetics for SULT1E1 Allozymes
Apparent K_m values (mean ± SE)

SULT1E1 Allozyme	Substrate	
	E2 (μM)	PAPS (nM)
Wild Type	0.030 ± 0.005	56 ± 2.9
Asp22Tyr	0.220 ± 0.03	240 ± 9.2
Ala32Val	0.044 ± 0.005	65 ± 4.2
Pro253His	0.097 ± 0.007	180 ± 7.5

N.B.
ALL FIGURES AND TABLE IN APPENDIX I PAGES 14-16.

Appendix II : Attachments

Abstracts and BBRC Manuscript

SULFATE CONJUGATION AND ESTROGEN-MEDIATED CARCINOGENESIS.
A.A. Adjei*, T.C.Wood*, P.C. Roche* and R.M. Weinshilboum.
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The carcinogenic effects of estrogens as a result of receptor-mediated mechanisms are well established, but a growing body of evidence indicates that estrogens may also be direct genotoxins. Specifically, catecholestrogens (CEs) such as 4-hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2) are estrogen metabolites that can be metabolically activated to quinones which can form depurinating DNA adducts. Prevention of the genotoxic effects of these estrogen metabolites can be achieved, in part, by the sulfate conjugation of CEs catalyzed by sulfotransferase (SULT) enzymes. Many human SULTs are genetically polymorphic, so inherited differences in the activities of these enzymes might contribute to the pathophysiology of breast cancer. Therefore, we have determined the activity of 13 recombinant human SULTs with both 4-OHE1 and 4-OHE2 as substrates. All but one of the enzymes studied could catalyze these reactions to varying degrees, but SULT1E1 had the highest affinity for the CEs, with apparent Km values of 0.31 μM for 4-OHE1 and 0.18 μM for 4-OHE2. We have also localized SULT1E1 to benign human breast tissue by immunohistochemical methods. These results indicate that individual variation in the sulfate conjugation of CEs catalyzed by SULTs may represent a risk factor for breast and other cancers.

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Sulfate Conjugation and Estrogen-Mediated Carcinogenesis.

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A growing body of evidence indicates that estrogens are direct genotoxins in addition to their well established role in carcinogenesis as a result of receptor-mediated mechanisms. Specifically, catecholestrogens (CEs) such as 2 and 4-hydroxyestrone (2-OHE1, 4-OHE1) and 2- and 4-hydroxyestradiol (2-OHE2, 4-OHE2) are estrogen metabolites that can be metabolically activated to form quinones. These quinones can cause oxidative DNA damage or they can form either stable or depurinating DNA adducts. Both oxidative damage and DNA adduct formation lead to carcinogenesis. Prevention of the genotoxic effects of these estrogen metabolites can be achieved, in part, by the metabolic conjugation of estrogens and CEs. Sulfotransferase (SULT) enzymes catalyze a major conjugation pathway in humans. Many human SULTs are genetically polymorphic, so inherited variation in estrogen or CE metabolic inactivation catalyzed by these enzymes might contribute to the pathophysiology of breast and other estrogen-dependent cancers. Therefore, we have determined the activity of 12 recombinant human SULTs with 2-OHE1, 4-OHE1, 2-OHE2, 4-OHE2 and with estrone (E1) and estradiol (E2) as substrates. All but one of the enzymes studied could catalyze the sulfation of these compounds. SULT1E1, originally described as an "estrogen SULT", had the lowest K_m values for all of the substrates tested. For example, apparent K_m values of SULT1E1 for 4-OHE1 and 4-OHE2, two procarcinogenic CEs capable of forming depurinating DNA adducts, were 0.31 and 0.18 μM, respectively. These results suggest that individual pharmacogenetic variation in the metabolism of estrogens and CEs catalyzed by SULTs may represent a risk factor for breast and other estrogen-dependent cancers.

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HUMAN ESTROGEN SULFOTRANSFERASE (SULT1E1) PHARMACOGENETICS: GENE RESEQUENCING AND FUNCTIONAL GENOMICS.

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SULT1E1 catalyzes the sulfate conjugation of steroid hormones including estrogens, catecholestrogens and the antineoplastic agent, 2-methoxyestradiol. Estrogen inactivation as a result of SULT1E1-catalyzed sulfation could also act as a protective mechanism against estrogen-dependent carcinogenesis. We previously cloned and characterized the human SULT1E1 cDNA and gene as steps toward pharmacogenetic studies. In the present experiments, we set out to determine whether common functionally significant SULT1E1 genetic polymorphisms might exit. Specifically, we "resequenced" the eight SULT1E1 exons, including splice junctions as well as portions of the 5′- and 3′- flanking regions using 120 DNA samples from 60 African-American and 60 Caucasian-American subjects. A total of approximately 730-kb of sequence was analyzed. Twenty-three SNPs were observed, including 3 non-synonymous cSNPs which altered the following encoded amino acids: Asp22Tyr in African- Americans and Ala32Val and Pro253His in Caucasian-Americans. Transient expression of constructs with these cSNPs in COS-1 cells showed significant decreases in SULT1E1 activity for Asp22Tyr and Ala32Val, with corresponding decreases in levels of immunoreactive proteins. However, there was a significant increase in activity for the Pro253His allozyme, but only a slight increase in level of immunoreactive protein. These observations suggest that ethnic-specific pharmacogenetic variation in SULT1E1-catalyzed sulfation of estrogens might contribute to the pathophysiology of estrogen-dependent disease in humans.

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Catecholestrogen Sulfation: Possible Role in Carcinogenesis

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A growing body of evidence supports the hypothesis that estrogens can be carcinogens as a result of their conversion to genotoxins after biotransformation to form the catecholestrogens (CEs) 2-hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 4-hydroxyestrone (4-OHE1) and 4-hydroxyestradiol (4-OHE2). CEs can then undergo further metabolism to form quinones that interact with DNA to form either stable or depurinating adducts. These events could potentially be interrupted by the sulfate conjugation of both the parent estrogens and/or the CEs. We set out to determine whether CEs can serve as substrates for sulfate conjugation, and—if so—which of the growing family of human sulfotransferase (SULT) isoforms are capable of catalyzing those reactions. We determined apparent K_m values for 10 recombinant human SULT isoforms, as well as the three most common allozymes for SULT1A1 and SULT1A2, with 2-OHE1, 2-OHE2, 4-OHE1, and 4-OHE2, and with the endogenous estrogens, estrone (E1) and 17β-estradiol (E2), as substrates. With the exception of SULT1B1, SULT1C1, and SULT4A1, all of the human SULTs studied catalyzed the sulfate conjugation of CEs. SULT1E1 had the lowest apparent K_m values, 0.31, 0.18, 0.27, and 0.22 μM for 4-OHE1, 4-OHE2, 2-OHE1, and 2-OHE2, respectively. These results demonstrate that SULTs can catalyze the sulfate conjugation of CEs, and they raise the possibility that individual variation in this pathway for estrogen and CE metabolism as a result of common genetic polymorphisms could represent a risk factor for estrogen-dependent carcinogenesis. © 2002 Elsevier Science (USA)

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Estrogen exposure is an important risk factor for breast cancer (1). The mechanism for estrogen-dependent carcinogenesis has been thought to result primarily from receptor-mediated events that stimulate cellular proliferation and promote tumor progression (2–4). However, there is also mounting evidence in support of a parallel and complementary mechanism that involves the conversion of estrogens to catecholestrogens (CEs) (5–7). These CEs can then undergo further metabolism to form CE-semiquinones and CE-quinones. The quinones are capable of binding to DNA to form potentially carcinogenic stable or depurinating DNA adducts (Fig. 1) (6–8). The 4-OHCE quinones are capable of forming depurinating adducts (7), and it is the 4-OHCEs that have been shown to be the most potent carcinogens in experimental systems such as the male Syrian Golden hamster kidney (9).

In addition to the metabolic "activation" pathway for estrogens and CEs that is depicted graphically in Fig. 1, there are also potentially protective metabolic pathways—pathways that often involve conjugation reactions. The best studied of those conjugation pathways is the O-methylation of CEs catalyzed by catechol O-methyltransferase (COMT). This genetically polymorphic enzyme (10–13) catalyzes the ring O-methylation of CEs. A series of studies have reported that subjects homozygous for the inherited trait of low levels of COMT activity appear to have an increased risk for the occurrence of breast cancer (14–16)—although contradictory results have also been reported (17, 18). Those observations imply that an inherited decrease in the ability to inactivate CEs or their metabolites by conjugation might represent a risk factor for estrogen-dependent genotoxicity and, therefore, for carcinogenesis.

Sulfation, like methylation, is a conjugation reaction. Furthermore, sulfate conjugation plays an important role in the biotransformation of steroid hormones—especially estrogens—as well as neurotransmitters, xenobiotics and many drugs (19). The majority of circulating estrogen in humans is sulfate conjugated (20). Therefore, unlike

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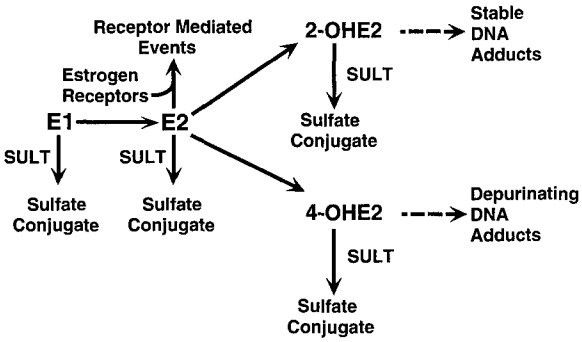

FIG. 1. Estrogen-induced carcinogenesis: Possible role for sulfation. The figure depicts schematically both receptor-mediated and direct genotoxic mechanisms for estrogen-induced carcinogenesis. It also shows potential sites for the SULT-catalyzed sulfate conjugation of estrogens and/or CEs.

COMT which can methylate CEs but not the "parent" estrogens E1 and E2, SULTs can also inactivate E1 and E2 by forming sulfate conjugates, thereby "diverting" these hormones from both receptor-mediated and genotoxic pathways leading to carcinogenesis (Fig. 1). Furthermore, many human SULTs are, like COMT, genetically polymorphic (21–25). Therefore, the possibility exists that individual, genetically determined variation in the sulfate conjugation of E1, E2 and/or the CEs, might also represent a risk factor for estrogen-dependent carcinogenesis.

As a step toward testing the hypothesis that individual variation in the sulfation of estrogens and CEs might represent a risk factor for carcinogenesis, we have performed substrate kinetic studies with ten human cytosolic SULT isoforms, as well as common allozymes for SULT1A1 and SULT1A2, to determine whether those enzymes can catalyze the sulfate conjugation of E1, E2 or the 2-OH and 4-OHCEs. Our understanding of the molecular biology and genomics of the SULT gene superfamily in humans has increased dramatically during the past decade (26, 27). That superfamily now includes at least three families in humans—the phenol or SULT1 family, the hydroxysteroid or SULT2 family, and SULT4A1—an enzyme with no known substrate (28). The SULT1 and SULT2 families each contain subfamilies with differing and overlapping substrate specificities. The dendrogram in Fig. 2 shows the relationship among the amino acid sequences encoded by all known human SULT genes. Figure 2 also lists the chromosomal locations of those genes and prototypic substrates for each human SULT isoform. In the course of the present studies, we observed that seven human SULT isoforms were capable of catalyzing the sulfation of both 2-OH and 4-OHCEs, that six isoforms catalyzed the sulfate conjugation of E2, but only two could utilize E1 as a substrate. These observations raise the possibility that individual variation in sulfation, like individual variation in methylation, might represent a risk factor for estrogen-dependent carcinogenesis.

MATERIALS AND METHODS

Reagents and chemicals. 2-OHE1, 2-OHE2, 4-OHE1 and 4-OHE2 were purchased from Steraloids (Wilton, NH). [^{35}S]-3′-Phosphoadenosine 5′-phosphosulfate ([^{35}S]-PAPS) (2.52 Ci/mole) was obtained from New England Nuclear Life Science Products (Boston, MA). E1 and E2 were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Recombinant human SULTs. cDNAs for human cytosolic SULTs as well as cDNAs encoded by the common variant alleles for human *SULT1A1* and *SULT1A2* were ligated into the eukaryotic expression vectors pCR3.1 or p91023(b). Inserts in these expression constructs were sequenced on both strands to verify DNA sequences. GenBank accession numbers for the open reading frame (ORF) sequences present in these expression constructs were L19999 for SULT1A1*1, L10819 for SULT1A1*2, U28169 for SULT1A2*1, U28170 for SULT1A2*2, X78282 for SULT1A2*3, L19956 for SULT1A3, D89479 for SULT1B1, U66036 for SULT1C1, U08098 for SULT1E1, U08024 for SULT2A1, U92314 for SULT2B1a, U92315 for SULT2B1b and AF1888698 for SULT4A1. COS-1 cells were transfected with each of the expression constructs using the DEAE-dextran method as described previously (22, 29–31) or with TransFast as suggested by the manufacturer (Promega, Madison, WI). Cytosol preparations from transfected cells were used as a source for recombinant human SULT proteins. Cytosol preparations from COS-1 cells transfected with "empty" vector that did not contain an insert served as controls for these assays.

SULT assays and substrate kinetic experiments. SULT enzyme activities were measured by modifications of the method of Foldes and Meek (32) as described Campbell *et al.* (33) and by Hernández *et al.* (34). This assay is based on the transfer of a radioactively labeled sulfonate group to an acceptor substrate, followed by precipitation of the unreacted radioactive donor molecule, ^{35}S-PAPS, with barium. The acceptor substrates studied were E1, E2, 2-OHE1, 4-OHE1, 2-OHE2 and 4-OHE2. Because SULTs often display profound sub-

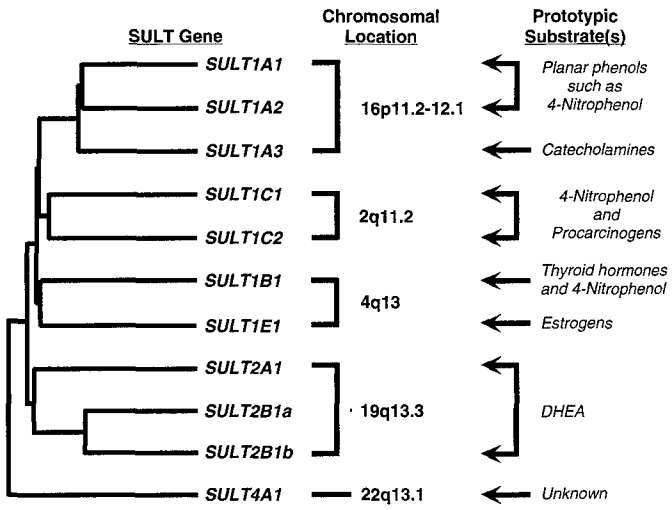

FIG. 2. Human SULT isoforms. The dendrogram shows the relationship among the amino acid sequences of known human SULT isoforms, the chromosomal location of genes encoding those isoforms and prototypic substrates for each isoform. The PILEUP program from the Wisconsin GCG package, Version 10, was used to create the dendrogram. DHEA is dehydroepiandrosterone.

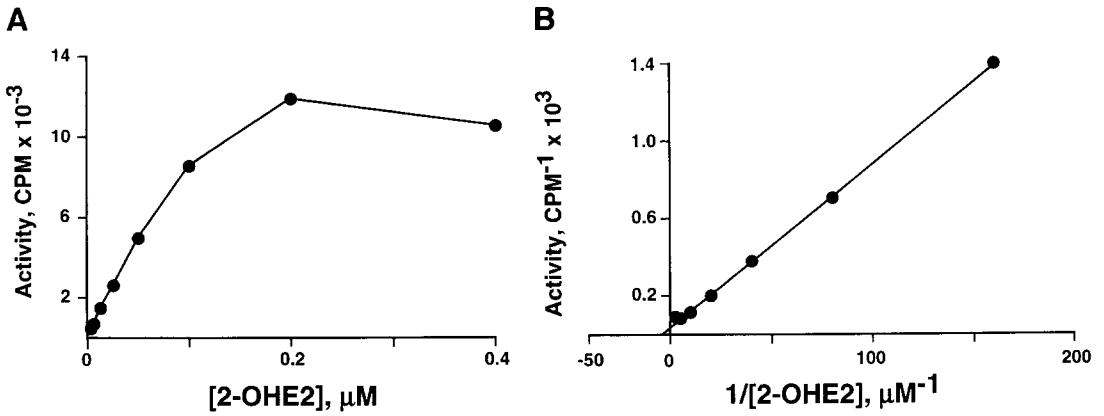

FIG. 3. Human SULT1E1 substrate kinetics with 2-OHE2 as a substrate. (A) Plot of activity versus [2-OHE2]. (B) Double inverse plot of 1/activity versus 1/[2-OHE2].

strate inhibition (19), the substrate kinetic studies were conducted in two steps. The initial step involved testing a wide range of acceptor substrate concentrations that varied from 10^{-3} to 10^{-8} M with steroid substrates dissolved in dimethyl sulfoxide. A second set of experiments was then performed with eight different concentrations of steroid substrate near the apparent K_m for that isoform. In all cases, a positive control in which a "prototypic" substrate at the optimal concentration for that particular isoform (i.e., 4-nitrophenol, dopamine, E1 or dehydroepiandrosterone, see Fig. 2) was included with each assay. The final concentration of PAPS in all experiments was 0.4 μM. Blank samples contained no sulfonate acceptor substrate. COS-1 cell cytosol from cells transfected with empty vector was also included with each set of assays to make it possible to correct for the contribution of endogenous SULT activity in the COS-1 cells to the activity measured. The endogenous SULT activity in the COS-1 cells constituted less than 5% of the activity observed in the presence of the optimal concentration of acceptor substrate. Those isoforms listed in Tables 1, 2, and 3 with "not detectable" (ND) activity all displayed either no or only minimal increases over endogenous activity levels (at most a 5–10% increase) in the presence of the optimal substrate concentration.

Protein assay. Protein concentrations were measured by the method of Bradford (35) with bovine serum albumin as a standard.

Data analysis. All assays were performed in triplicate, and values reported are averages for those three determinations. Apparent Michaelis constants were calculated with the method of Wilkinson (36) using a computer program written by Cleland (37). The variance measure listed is the SEM.

RESULTS AND DISCUSSION

We set out to test the hypothesis that sulfation might contribute to the biotransformation of CEs, and that different human SULT isoforms might participate in or contribute to that process. Therefore, a total of fourteen recombinant human SULT proteins were used to perform substrate kinetic experiments with the parent estrogens E1 and E2, as well as 2-OH and 4-OHCEs derived from those two steroid hormones. All assays were performed under optimal conditions for the specific SULT isoform being studied using a modification of the assay of Foldes and Meek (32). Because SULTs are subject to profound substrate inhibition—often resulting in a lack of detectable activity at high substrate concentrations (19)—two sets of experiments were performed for each substrate studied. The purpose of the initial experiment was to determine an approximate concentration range for use in the definitive studies. In each case, the initial experiment involved studies performed with 10-fold serial dilutions of acceptor substrate that ranged from 10^{-3} to 10^{-8} M. In the second—definitive—experiment, eight different concentrations of 2-fold serial dilutions of the same substrate in a range that resulted in maximum activity during the initial experiment were used to determine apparent K_m values. A typical curve for one substrate, 2-OHE2, as well as a double inverse plot of those same data, is shown in Fig. 3. By applying this strategy, it was possible to calculate apparent K_m values for E1, E2 as well as 2-OH and 4-OHCEs derived from those compounds (Tables 1, 2, and 3).

Apparent K_m values of recombinant human SULTs for 2-OH and 4-OHCEs are listed in Tables 1 and 2, while those for the parent estrogens E1 and E2 are listed in Table 3. A series of generalizations were possible on the basis of these data. First, it was clear that SULT1E1 was the isoform that displayed the lowest apparent K_m values and, thus, the highest affinity, for all of the estrogen compounds that we studied. That observation is compatible with the fact that SULT1E1 was originally named "estrogen sulfotransferase" (29). Second, SULT1B1, SULT1C1 and SULT4A1 failed to catalyze the sulfation of any of the six estrogen compounds tested (Tables 1, 2, and 3). Those observations were also not surprising, since there is no known substrate for SULT4A1, (28), SULT1C1 has been reported to only catalyze the sulfate conjugation of 4-nitrophenol and N-hydroxy-2-acetylaminofluorene (23, 38, 39), and SULT1B1 has been reported to only catalyze the sulfation of 4-nitrophenol and thyroid hormones (40, 41). Third, there were very large differences among isoforms in apparent K_m values for any

TABLE 1

Apparent K_m Values for Human SULT Isoforms and Allozymes with 2-OHE1 and 2-OHE2 as Substrates

Estrogen-induced carcinogenesis K_m values for 2-OHE1 (E2)		
SULT isoform or allozyme	Apparent K_m values μM ± SEM	
	2-OHE1	2-OHE2
1A1*1	5.3 ± 1.3	2.5 ± 0.2
1A1*2	3.6 ± 2.8	11.6 ± 0.5
1A1*3	3.6 ± 0.4	17.3 ± 2.0
1A2*1	19.6 ± 2.4	10.7 ± 1.6
1A2*2	31.5 ± 3.2	485 ± 75.1
1A2*3	8.9 ± 1.2	40.8 ± 2.6
1A3	188 ± 4.0	81.8 ± 6.9
1B1	ND	ND
1C1	ND	ND
1E1	0.27 ± 0.08	0.22 ± 0.03
2A1	12.7 ± 1.3	2.4 ± 0.1
2B1a	6.7 ± 1.5	3.3 ± 0.4
2B1b	3.3 ± 1.5	2.5 ± 0.1
4A1	ND	ND

Note. ND, little or no detectable activity. Values are mean ± SEM ($N = 3$).

TABLE 3

Apparent K_m Values for Human SULT Isoforms and Allozymes with E1 and E2 as Substrates

Estrogen-induced carcinogenesis K_m values for E1 and E2		
SULT isoform or allozyme	Apparent K_m values μM ± SEM	
	E1	E2
1A1*1	ND	31.3 ± 6.2
1A1*2	ND	84.6 ± 24.3
1A1*3	ND	21.1 ± 2.9
1A2*1	ND	28.6 ± 3.8
1A2*2	ND	145 ± 21
1A2*3	ND	18.3 ± 2.2
1A3	ND	ND
1B1	ND	ND
1C1	ND	ND
1E1	0.11 ± 0.1	0.029 ± 0.01
2A1	11.2 ± 0.9	12.4 ± 0.9
2B1a	ND	84.3 ± 7.6
2B1b	ND	60.9 ± 10.0
4A1	ND	ND

Note. ND, little or no detectable activity. Values are mean ± SEM ($N = 3$).

given substrate. Finally, there were also differences in apparent K_m values among common allozymes, different amino acid sequences encoded by variant alleles, for *SULT1A1* and *SULT1A2*—the two isoforms for which recombinant allozymes were available.

When comparisons were made between the two classes of CEs studied, the 2-OH and 4-OHCEs, K_m values for specific isoforms or allozymes were often lower for the 2-OH than for the 4-OH CEs—occasionally by an order of magnitude (Tables 1 and 2). That observation was of potential importance for several reasons. First, in most model systems the 4-OHCEs are more carcinogenic than are the 2-OHCEs (9, 42, 43). Furthermore, the methyl derivative of 2-OHE2 is an antiangiogenic compound that is presently undergoing testing as an antineoplastic agent (44, 45) and 2-methoxy E2 itself has been reported to undergo sulfation (46). Therefore, the potential effects of sulfation on estrogen-dependent carcinogenesis might ultimately prove to be even more complex than those of COMT, an enzyme that requires the catechol structure and, as a result, plays no role in the metabolism of E1, E2 or conjugated metabolites of E1, E2 or the CEs. It should also be noted that preliminary reports have already appeared which indicate that CE sulfation may play a role in the pathophysiology of one estrogen-dependent tumor, breast cancer (47–49). The **2* variant allele of *SULT1A1* encodes an allozyme with significantly decreased activity, and *SULT1A1*2* has been reported to represent a risk factor for breast cancer (49)—in theory as a result of the decreased sulfation of estrogens and/or CEs (see Fig. 1). However, another study reported a lack of association of *SULT1A1* genotype with risk for breast cancer (50).

Our substrate kinetic studies of the endogenous parent estrogen compounds E1 and E2 showed that E1 differed significantly from E2 as a substrate for SULT-catalyzed sulfation (Table 3). Among the SULT isoforms studied, only SULT1E1 and SULT2A1 utilized E1 as a substrate, while—with the exception of SULT1A3, 1B1, 1C1, and 4A1—all isoforms were ca-

TABLE 2

Apparent K_m Values for Human SULT Isoforms and Allozymes with 4-OHE1 and 4-OHE2 as Substrates

Estrogen-induced carcinogenesis K_m values for 4-OHE1 (E2)		
SULT isoform or allozyme	Apparent K_m values μM ± SEM	
	4-OHE1	4-OHE2
1A1*1	53.8 ± 2.1	44.0 ± 5.2
1A1*2	53.3 ± 9.3	23.4 ± 3.7
1A1*3	40.4 ± 4.4	6.3 ± 0.1
1A2*1	17.1 ± 2.0	27.9 ± 0.7
1A2*2	65.1 ± 9.4	42.4 ± 2.7
1A2*3	5.1 ± 0.8	7.6 ± 2.0
1A3	32.4 ± 3.7	476 ± 94
1B1	ND	ND
1C1	ND	ND
1E1	0.31 ± 0.13	0.18 ± 0.06
2A1	20.4 ± 1.0	41.5 ± 19.5
2B1a	20.7 ± 1.8	45.2 ± 13.0
2B1b	13.6 ± 0.4	17.5 ± 1.0
4A1	ND	ND

Note. ND, little or no detectable activity. Values are mean ± SEM ($N = 3$).

TABLE 4

Apparent K_m and V_{max} Values for Human SULT1E1 with E1, E2, 2-OHE1, 2-OHE2, 4-OHE1, and 4-OHE2 as Substrates

	SULT1E1 substrate kinetics		
Substrate	K_m, μM	V_{max}, units/mg protein	V_{max}/K_m
E2	0.029 ± 0.01	6.88 ± 0.05	237
E1	0.11 ± 0.1	4.13 ± 0.14	37.5
4-OHE2	0.18 ± 0.06	6.04 ± 0.12	33.6
2-OHE2	0.22 ± 0.03	4.77 ± 0.22	21.7
4-OHE1	0.31 ± 0.13	2.35 ± 0.004	7.6
2-OHE1	0.27 ± 0.08	1.61 ± 0.18	6.0

Note. "Units" of enzyme activity represents nmol/h. Values are mean ± SEM (N = 3).

pable of catalyzing E2 sulfation (Table 3). These observations are of potential functional importance since sulfate conjugated E1 is a major metabolite of that steroid hormone in humans (20, 51), and there are functionally significant genetic polymorphisms for human SULT2A1 and 1E1 (24, 25)—the only two isoforms capable of catalyzing E1 sulfation.

The data listed in Tables 1, 2, and 3 provide information with regard to which human SULT isoforms are capable of catalyzing the sulfate conjugation of estrogens and CEs. However, since the recombinant proteins used to perform those experiments were derived from a series of independent COS-1 cell transfections, it was not possible to compare V_{max} data among substrates. Since SULT1E1 had the lowest apparent K_m values for all of the estrogen and CE substrates tested (Tables 1, 2, and 3), we performed a single large transfection of COS-1 cells with the SULT1E1 expression construct and tested all of the compounds studied with that single enzyme preparation. By using that approach, it was possible to directly compare apparent K_m and apparent V_{max} data for all substrates with this important isoform (Table 4). Although K_m values of SULT1E1 for E1 and E2 were lower than those for the CEs, the V_{max}/K_m ratio for 4-OHE2, a carcinogen (Table 4), was roughly comparable to that for E1. Of the six substrates studied, E2 had the highest V_{max}/K_m ratio, 237, and 2-OHE1 had the lowest, 6.0 (Table 4). These data served to focus attention on SULT1E1 as an isoform worthy of future study as a possible factor involved in variation of estrogen-dependent carcinogenesis—especially since functionally significant genetic polymorphisms for this isoform have been described recently (25).

In summary, the present study indicates that individual variation in sulfate conjugation is one factor that could contribute to variation in risk for estrogen-dependent carcinogenesis. Unlike the methylation of CEs catalyzed by COMT, sulfation could also result in variation in the availability of the parent estrogens, E1 and E2 (see Fig. 1). Therefore, variation in sulfate conjugation could potentially affect both receptor-mediated and genotoxic mechanisms for estrogen-dependent carcinogenesis (Fig. 1). The next step in testing that hypothesis will require the characterization of common, functionally significant genetic polymorphisms within genes that encode all human SULT isoforms which participate in estrogen or CE conjugation. That process has already begun (22–25). It will then be necessary to determine whether any of those functional polymorphisms might represent risk factors for the occurrence of estrogen-dependent tumors. The present experiments represent a step toward achievement of that ultimate goal.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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